

A STUDY OF TRANSAMINATION IN AMPHIBIA

by

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To Olivetti, for their kind loan of a typewriter.

Z.R. GOLDSTEIN.

I declare that the thesis which is herewith submitted
for the degree of Master of Science in the University
of the Witwatersrand is entirely my own work and that
it has not been previously submitted for a degree in
any other University.

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List of Abbreviations used in the Text.

G.O.T.	Glutamic oxaloacetic transaminase or aspartate aminotransferase.
G.P.T.	Glutamic pyruvate transaminase or alanine aminotransferase.
Asp.	Aspartic acid.
Ala.	Alanine.
Glu.	Glumatic acid.
α -kg.	α -ketoglutaric acid.
DNP.	Dinitrophenyl hydrazine.
ATP.	Adenosine triphosphate.
TCA.	Trichloroacetic acid.

INTRODUCTION

A. NITROGEN METABOLISM IN AMPHIBIA.

1. Ammoniotelism, Ureotelism and Uricotelism.

Waste nitrogen, arising as an end product of amino acid metabolism, is excreted by vertebrates in one of three forms, viz. ammonia in ammoniotelic animals, urea in ureotelic animals and uric acid in uricotelic animals.

2. Adaptation to Environment.

Delauney (1931, 1934) was the first person to suggest that the nature of the end product of nitrogen metabolism depended on the environment of the animal. Ammonia is the primary end product of amino acid catabolism and therefore the most economical way of eliminating waste nitrogen would be the direct excretion of ammonia. However a major disadvantage is its toxicity and ammoniotelism is therefore only feasible in animals which have an abundant water supply, thereby allowing the ammonia to be excreted directly into the surrounding water. In this way it would not have to be stored in the tissues. Of course,

this could only occur in animals in which water loss was not important. Thus aquatic animals like the Pipidae (of which Xenopus laevis is an example) are predominantly ammoniotelic (Cragg et al., 1961), excreting 62% of their waste nitrogen as ammonia (the rest being urea).

Species which are not completely aquatic cannot excrete the ammonia quickly enough, and a build-up of this substance is unacceptable because of its toxicity to the central nervous system. Delauney thus suggested that terrestrial animals developed the power to manufacture urea or uric acid as a non-toxic waste product.

Richter & Dawson (1948) suggested that uric acid, being relatively insoluble, would be advantageous to animals with restricted water supply e.g. desert species. Uric acid can be dehydrated and excreted as a solid with negligible water loss.

Some interesting adaptations of the mode of excretion to environment have occurred among the Amphibia. Most Amphibia are only semi-aquatic and spend a large proportion of their time on land, becoming completely aquatic only during the mating season when they return to water to mate and lay their eggs.

Some species are more water dependent than others, e.g. Rana species are restricted to moist surroundings and are often found in water. All species of Rana which have been studied are ureotelic (Cragg et al., 1961), but they still excrete a significant amount of ammonia. On the other hand Bufo is more terrestrial than Rana, and significantly excretes less ammonia.

Amphibia were the first class of vertebrates which successfully colonized land, and the biochemical characteristics of the Amphibia are thus the origin of adaptations to terrestrial environment in the whole phylum (Balinsky, 1970, P. 519). Needham (1931) related this ability to survive on land to the development of a non-toxic nitrogenous waste product, but explained that this represented a considerable waste of chemical energy. Ammonia is by far the most economical excretory end product, having a lower free energy of formation than urea or uric acid. Moreover, the formation of urea and uric acid requires the maintenance of high levels of a number of enzymes which also consumes energy. In a terrestrial environment, however, the toxicity outweighs its economy of energy.

Thus while excretion of urea is an adaptation to a terrestrial environment, it is extremely interesting to find that a completely aquatic animal like Xenopus does

excrete some urea, and has all the enzymes of the urea cycle. Except for arginase, these exhibit lower activities per unit weight of liver than those from ureotelic Amphibia such as Rana. (Balinsky & Baldwin, 1961; Balinsky & Silver, 1961.)

Xenopus adapts to water shortage by aestivating in the mud of dried pools during the dry season. Under these conditions urea is synthesized and stored in the tissues. Ten- to twenty-fold increases in tissue urea have been observed. On the return of favourable conditions the stored urea is excreted and the animal becomes ammoniotelic again. These conditions can be induced experimentally by keeping the animals out of water, or by restricting their water supply osmotically by keeping them in 0.15M NaCl, which is hypertonic to them. The production of urea is due to an increase in the level of the first of the urea cycle enzymes, carbamoyl phosphate synthetase. (Balinsky et al, 1967b).

Aestivation also causes a two- to three-fold increase in concentration of plasma and tissue ammonia (Balinsky, 1970, P. 560). This may be significant since ammonia accumulation may stimulate urea synthesis, as it is a starting point for urea synthesis in the liver. Thus the effect of dehydration is to channel waste nitrogen from ammonia

to urea, thereby limiting the accumulation of toxic ammonia in the tissues.

3. Nitrogen Excretion during Metamorphosis.

The larvae of all species of Amphibia are aquatic and develop in water until metamorphosis, the climactic transformation, both morphological and biochemical, which accompanies transition to terrestrial life. During this time, the young animals re-enact as it were, the evolutionary changes undergone by their ancestors when they emerged on land (Balinsky, 1970, P. 520).

Since metamorphosis in Amphibia is often accompanied by a change in environment, it is to be expected that changes in the excretory product would be observed. Being aquatic, the larval forms all excrete ammonia and the change to a terrestrial mode of life is accompanied by a corresponding change from ammonotelism to ureotelism (Munro, 1939, 1953).

Thus the relationship between environment, metamorphosis and nitrogenous end product illustrates the advantages of excreting ammonia to aquatic animals, and of excreting urea to terrestrial animals.

4. Synthesis of Urea

1. The Krebs Urea Cycle

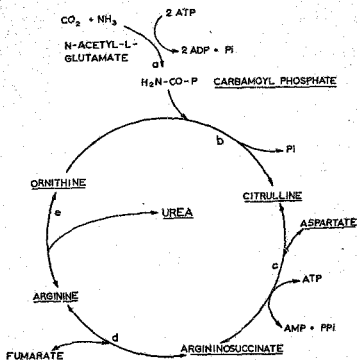
The enzyme mechanism for urea synthesis in mammals was elucidated by Krebs & Henseleit (1932). They suggested that urea was formed by a cyclic process starting with ornithine and proceeding to the eventual production of urea and regeneration of ornithine (Figure 1a P. 7).

The experiments performed by the above authors in mammals were repeated by Manderscheid (1933) in liver slices of Rana esculenta. She obtained similar results and concluded that the cyclic reaction sequence suggested by Krebs & Henseleit was also valid for Amphibia.

The activities of the individual enzymes have been studied by a large number of workers in a wide variety of vertebrate species. An early experiment by Clementi (1914) demonstrated that a general correlation exists between urea synthesis and the activity of

Fig. 1a.

THE KREBS UREA CYCLE



The enzymes catalysing the reactions are as follows:

- Carbamoyl phosphate synthetase
- Ornithine transcarbamoylase
- The condensing enzyme
- The cleavage enzyme
- Arginase

arginase. More recently Brown & Cohen (1960), as well as various other workers (summarised by Balinsky, 1970, P. 564) published a survey of all the urea cycle enzymes in a large number of species. Their results showed that production of urea is confined to the livers of Amphibia, only arginase being found to any large extent in the kidneys of these animals.

Brodsky et al (1965) subsequently showed that some urea was synthesised in the kidney of Rana catesbeiana but it was later found that the urea cycle was not functional in Rana kidneys since one of the enzymes, argininosuccinase, was absent. Thus it was postulated that the urea in the kidney was derived either from the hydrolysis of arginine resulting from protein breakdown, or from allantoinic acid, which is a breakdown product of uric acid.

Thus it can be stated that the production of urea as an end product of amino acid metabolism is confined to the liver of Amphibia.

In mammals urea synthesis and ammonia production are closely linked (Kamin & Handler, 1951). These

workers found that urea synthesis consisted of a succession of two, possibly interrelated, processes. The first is the removal of nitrogen from amino acids, followed by synthesis of urea from that nitrogen by the Krebs Henseleit mechanism. They found the rate of urea formation from arginine to be greater than from any amino acid except glutamine. Moreover they showed that no free ammonia accumulated during the deamination of amino acids in the liver indicating that the ammonia so formed was immediately converted to urea, thereby avoiding any accumulation of this toxic substance.

ii. Levels of the Urea Cycle Enzymes.

Brown & Cohen (1960) measured the activities of carbamoyl phosphate synthetase and ornithine transcarbamoylase by estimating citrulline production and the activities of the arginine synthetase system and arginase by estimating urea produced.

Mora et al (1965) studied the urea cycle enzymes in several animals, showing that the levels of carbamoyl phosphate synthetase, ornithine transcarbamoylase and the arginine synthetase system all correlate with the amount of nitrogen

excreted as urea. They suggested the existence of a common regulatory mechanism for the first four enzymes of the urea cycle.

However, Balinsky (1970) has pointed out that this postulate is not always applicable. Carbamoyl phosphate synthetase and ornithine transcarbamoylase show much more variation than argininosuccinate synthetase, therefore if there is a common control mechanism, it is not the only one (Balinsky, 1970, P. 564).

Brown & Cohen (1960) suggested that argininosuccinate synthetase (the condensing enzyme) was the rate limiting enzyme since they found it always had the lowest in vitro activity. They showed that all enzymes of the urea cycle were present at higher levels in the livers of ureotelic animals than in those animals which do not excrete large quantities of urea. It follows therefore, that urea excretion is not correlated so much with the activity of arginase, as suggested by Clementi (1914), as with the activity of all the enzymes of the cycle.

It is necessary to bear in mind, however, that in vitro conditions might not resemble those of the intact cell and a comparative approach is therefore more reliable. In comparing the same enzyme in different species, and using

the same techniques, it is reasonable to assume that the enzyme suffers the same disruptions in each case and hence relative activities provide a stronger basis for a theory than absolute values.

Thus, although argininosuccinate synthetase always shows the lowest in vitro activity, it need not be the rate limiting enzyme. Similar activities are found in ureotelic Rana and ammoniotelic Xenopus, and an increase in activity accompanies metamorphosis in both animals, although Xenopus remains ammoniotelic while Rana changes to ureotelism.

If one compares the urea cycle enzymes in different species, carbamoyl phosphate synthetase, the first enzyme of the cycle, seems to give the best correlation with ureotelism. It is also the only enzyme to be raised when Xenopus becomes temporarily ureotelic (P.4). A comparison of this enzyme in Xenopus, the ureotelic Amphibian Rana angolensis and the ureotelic Mammal Rattus norvegicus shows that the level in ureotelic animals is some thirty times greater than in Xenopus. This supports the idea that in carbamoyl phosphate synthetase lies the key to urea synthesis. The six-fold increase in its activity in aestivating, urea-producing Xenopus lifts the enzyme level almost to that of the ureotelic animals (Balinsky et al 1967a).

Ornithine transcarbamoylase activity is also thirty to forty times greater in ureotelic animals than in Xenopus. Although no significant increase was found in aestivating frogs, the possibility that this enzyme has a role in the regulation of urea synthesis cannot be ruled out.

iii. The Effect of Environment on the Urea Cycle Enzymes.

As mentioned on P. 4, Xenopus adapts to conditions of water shortage by synthesising urea, which is accumulated in the tissues (Balinsky et al 1961, 1967b). This can be seen as an adaptive mechanism since, due to the toxicity of ammonia, only accumulation of urea would enable the animal to survive drought or to undertake land journeys.

While the accumulation of ammonia in the liver may, at least initially, be responsible for accelerated synthesis of urea, another important factor is the increase in level of some of the enzymes of the urea cycle. Significantly, it is the first enzyme of the cycle, carbamoyl phosphate synthetase, which is raised six-fold on aestivation and returns to normal after return to water. All other enzymes remain unaffected (Balinsky et al, 1967b, Janssens & Cohen, 1968b). This suggests firstly, an important role for carbamoyl phosphate synthetase in the control

of urea synthesis; being the first enzyme of the cycle it is plausible that it should be rate limiting.

Secondly, the fact that there is no increase in arginase activity under conditions of water restriction, as shown by Balinsky & Baldwin (1962), proves the suggestion by Dolphin & Frieden (1955), that arginase is the key enzyme of the cycle, to be incorrect. Moreover Balinsky & Baldwin showed that the arginase activity during water shortage is more than sufficient to account for the urea synthesized.

The effect of environment on the urea cycle enzymes of Rana cancrivora was studied by Balinsky & Dicker (1971). This is a crab eating frog with the unusual ability to survive in fresh and salt water. It adapts to salt water by raising the osmotic pressure of the blood through an increase in its blood urea concentration. The above authors found that all the urea cycle enzymes were elevated during adaptation to high saline concentration.

iv. The Effect of Metamorphosis on Urea Cycle Enzymes.

The change from an aquatic to a terrestrial mode of life, accompanied by a change from ammoniotelism to ureotelism, occurs in all Amphibia except Xenopus, and one would

expect therefore that the activity levels of all the urea cycle enzymes would remain constant in the latter, while those of Rana and Bufo should increase.

Brown, Brown & Cohen (1959) studied ureogenesis in Rana catesbeiana and found that during metamorphosis the percentage nitrogen excreted as urea rose rapidly. They also found that the activity levels of all the urea cycle enzymes increased together during metamorphosis, rising to the adult level. Similar results have been found in Rana angolensis (Balinsky et al., 1967a).

Munro (1939, 1953) and Balinsky et al. (1967b) found no increase in the percentage nitrogen excreted as urea during the development of Xenopus laevis although there was a considerable increase in the overall amount of nitrogen excretion at metamorphosis. This is probably due to the protein degradation arising from the resorption of the tail. However the percentage nitrogen excreted as urea is generally higher in the adult.

Balinsky (personal communication) measured the levels of the five urea cycle enzymes, carbamoyl phosphate synthetase, ornithine transcarbamoylase, argininosuccinate synthetase, argininosuccinate lyase and arginase at different stages of development of Xenopus laevis. Only carbamoyl phosphate

synthetase increased during the climax of metamorphosis. All the enzymes were higher, however, in the adult frogs than in the young froglets.

v. Control of the Urea Cycle.

Thyroxine is known to be the hormone directly inducing metamorphosis, and producing the changes in enzyme levels associated with it. (Dolphin & Frieden, 1955, Shambaugh et al, 1969 and Balinsky et al, 1969.) During the metamorphosis of Xenopus laevis, a peak of thyroid activity is observed immediately preceding the metamorphic climax. Unlike other Amphibia, Xenopus does not become terrestrial at metamorphosis, and the percentage of nitrogen excreted as urea does not change.

On treatment with thyroxine, Balinsky (personal communication) found a two-fold increase in the level of carbamoyl phosphate synthetase in premetamorphic Xenopus tadpoles, but there was no significant effect on the other enzymes. It appears therefore, that either the systems controlling the levels of these enzymes do not become sensitive to thyroxine until well after metamorphosis, or that another hormone is responsible for the elevation of the enzyme levels in the post-metamorphic animals.

The effect of thyroxine on the first enzyme of the cycle, carbamoyl phosphate synthetase, gives additional support to the postulation that it is the rate limiting enzyme of the cycle (See P. 11).

5. Formation of Ammonia.

The livers of higher vertebrates contain enzymes for nitrogen breakdown. Thus one would expect that if nitrogenous compounds are metabolised in the liver to form excretory ammonia, one would find ammonia in the blood en route to the kidneys. However, this is not the case. The ammonia formed in the liver is converted to the less toxic urea or uric acid, which is then taken to the kidney, while excretory ammonia is formed in the kidneys. Xenopus laevis is exceptional in that there is a significant amount of ammonia in the blood and tissues, and some excretory ammonia does appear to be formed in the liver, although the bulk is formed in the kidneys. Thus ammonia arising from deamination of alanine forms urea in the liver and remains as ammonia in the kidney. (Balinsky & Baldwin, 1961.) Subsequently Unsworth et al (1969) provided evidence for the direct excretion of plasma ammonia by Xenopus.

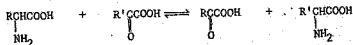
Although it is always in contact with water, the skin of Xenopus eliminates less than 15% of the ammonia excreted. (Balinsky & Baldwin, 1961.) Larval Amphibia have a third route of excretion available to them, viz. through the gills.

B. TRANSAMINATION.

1. General Background.

i. The scope of transamination.

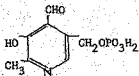
In general, the transamination reaction can be described as a transfer of an amino group from an amino acid to a keto acid, thereby forming new amino and keto acids:



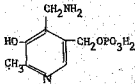
As can be seen from the above equation, no free ammonia is involved.

The mechanism of the reaction requires pyridoxal phosphate, a derivative of vitamin B₆, which has been found as a component of all transaminases studied and is therefore essential for the reaction to occur. In fact, trivalent cations such as Al³⁺, together with pyridoxal phosphate have been shown to catalyse transamination in vitro in the absence of enzymes.

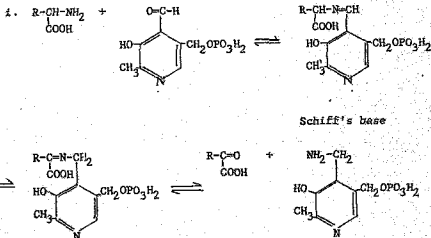
Pyridoxal phosphate



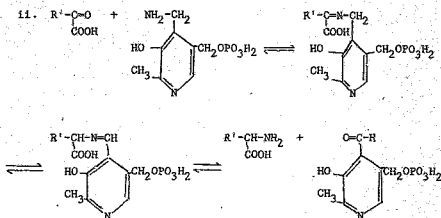
Pyridoxamine phosphate



The pyridoxal phosphate-enzyme complex reacts with the first amino acid forming a Schiff's base; this rearranges to give pyridoxamine phosphate-enzyme and an α -keto acid. The pyridoxamine phosphate then reacts with another keto acid to give a second amino acid and restores the pyridoxal phosphate-enzyme complex:



Schiff's base



The pyridoxal phosphate appears to be bound to the enzyme by groups other than those required for enzyme activity.

Enzymic transamination was first reported by Braunstein & Kritzman (1937). They observed the transfer of amino groups from α -amino acids to α -keto acids in pigeon breast muscle. Further work done independently by Braunstein (1947) and Cohen (1951) led them to conclude that only three amino acids were transaminated, viz. alanine, aspartic acid and glutamic acid:

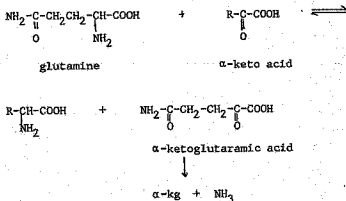
- i. $\text{Ala} + \alpha\text{-kg} \rightleftharpoons \text{pyruvate} + \text{Glu}$
- ii. $\text{Asp} + \alpha\text{-kg} \rightleftharpoons \text{oxaloacetate} + \text{Glu}$
- iii. $\text{Ala} + \text{oxaloacetate} \rightleftharpoons \text{Asp} + \text{pyruvate}.$

Since 1950 however, studies by several independent investigators led to the conclusion that nearly all natural amino acids are transaminated.

Cammarata & Cohen (1950) established the wide scope of transamination in rabbit liver, and in the liver, heart and kidney of the pig. They showed that twenty-two amino acids take part in transamination reactions to some extent, each probably catalyzed by a separate enzyme, and that the reaction is accelerated by pyridoxal phosphate and inhibited by ammonia. The same authors (Cammarata & Cohen, 1951) then studied the stability and specificity of the lyophilized glutamic oxaloacetate transaminase, finding it to be active over a two to three week period after which it declined rapidly. The enzyme was found to be fairly specific, showing no ability to transaminate various other amino acids.

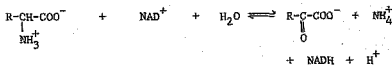
Meister (1950, 1952, 1954) reported that a number of amino acids were shown to transaminate with α -kg to give glutamate and the corresponding α -keto analogue, and that the α -amides of glutamine and asparagine were enzymically transaminated to their α -keto analogues. Later work showed that transamination is not restricted to reactions in which dicarboxylic α -amino acids or α -keto acids participate; D-amino acids, aldehydes and ω -amino acids may also be involved, as well as monocarboxylic α -amino acids and keto acids. In mammalian tissue however, no evidence has been found for D-amino acid transamination.

Transamination with glutamine and asparagine may be represented as transamination followed by hydrolysis of the α -keto acid ω -amide, which is catalysed by a specific transaminase-amidase:



The amide transaminase reaction is irreversible because of the rapid deamination of the α -keto acid amide.

Because of the generally low activity of L-amino acid oxidase which has been reported in the livers of mammals (and probably Amphibia as well (Balinsky & Baldwin, 1962)) a considerable amount of amino acid nitrogen may undergo the following transdeamination:



11. The Role of Transamination in General Metabolism

Transamination allows for a redistribution of nitrogen and is involved in the biosynthesis and/or degradation of most amino acids. Transamination reactions provide a means of converting alanine, glutamic and aspartic acid to the α -keto acid intermediates of the Citric Acid Cycle, and are involved in urea formation and ammonia metabolism. Thus:

- a. Transamination is the initial step in the degradation of valine, isoleucine, leucine, phenylalanine and tyrosine.
- b. It is the final step in the synthesis of isoleucine, valine, leucine, phenylalanine and tyrosine in microorganisms.
- c. It is involved in the synthesis of aspartate, alanine, histidine, lysine, ornithine, (which gives rise to arginine) and serine.
- d. The formation of phenylacetyl glutamic acid in humans indicates that phenylacetate is formed from phenylalanine via phenylpyruvate, which is formed by transamination.
- e. Transamination of amino acids with α -kg gives rise to glutamic acid which can give aspartic acid and hence urea.

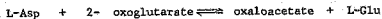
In the course of evolution the animal organism lost the ability to synthesize the carbon chain of certain α -keto acids. The corresponding amino acids cannot be formed by transamination reactions and must be supplied in the diet.

The relationship between transamination and urea production is an interesting one and forms part of the present study. Since aspartate combines with citrulline to give argininosuccinic acid, and hence urea, transamination of oxaloacetate with glutamate is an important channel into the urea cycle, and will be discussed under section 2(4) (P. 39).

iii. Purification and Properties of Transaminases

A large amount of work has been done on the purification and properties of various transaminases, particularly glutamic aspartate, glutamic alanine and glutamic tyrosine transaminases. Glutamic aspartate transaminase is particularly important because the corresponding ketoacids (oxaloacetate and α -kg) participate in the Krebs cycle.

Glutamic aspartic transaminase catalyses the reaction:



It is called in full: L-Aspartate:2 oxoglutarate aminotransferase, E.C.2.6.1.1., or, more commonly Aspartate

aminotransferase. It is abbreviated as G.O.T. (Glutamic oxaloacetic transaminase).

This enzyme was extensively purified from pig heart by Lis (1958, 1959) using techniques such as precipitation with acetone, ion exchange chromatography on carboxymethyl cellulose and zone electrophoresis. Many early workers found that their preparations led to dissociation of the enzyme which had then to be reactivated by addition of pyridoxal phosphate, but Lis obtained the holoenzyme purified fifty fold. Lis found acetone precipitation to be a suitable method for removing impurities prior to application of the material to the ion exchange column. Since the enzyme seemed to be quite insensitive to denaturation in the presence of fairly high acetone concentrations and at temperatures as high as 10° - 15° C it retains full activity for some hours at room temperature in 30% acetone.

Nisonoff et al (1952) studied the kinetics and mechanism of the G.O.T. reaction, the initial rates being measured spectrophotometrically by following changes in oxaloacetate concentration. Phosphate buffer salts were shown to have a marked activating effect.

A wide study of the properties of the enzyme from pig heart, its purification, assay, mechanism and the influence of pH

on the absorption spectrum and activity was done by Jenkins & Sizer (1959, 1960). They prepared two active forms of the enzyme - one with bound pyridoxal phosphate and the other with bound pyridoxamine phosphate - and studied their interconversion by transamination with appropriate substrates. They found that two residues of pyridoxal phosphate were bound per molecule of molecular weight $110,000 \pm 11,000$. The pH optimum of the enzyme was found to be around about pH 8.0; however the assay procedure was unsuccessful above pH 8.5 because of excessive breakdown of oxaloacetate to pyruvate. The enzyme behaves as a pH indicator, changing from bright yellow below pH 7 ($\lambda_{\text{max}} = 430 \text{ m}\mu$) to colourless at pH 8 ($\lambda_{\text{max}} = 362 \text{ m}\mu$). Above pH 12 the solution turns yellow again due to liberation of pyridoxal phosphate from the denatured enzyme. Tests on the heat stability of the enzyme showed it to be less stable in the presence of aspartate than with α -kg. The protonated enzyme was found to complex with certain dicarboxylic acids which inhibit its catalytic activity, because the acids are substrate analogues.

These findings were supported and extended by the work of Velick & Vavra (1962) who made a survey of the properties, kinetics, purification, spectra and inhibitors of the enzyme in pig heart. They reported that the purified

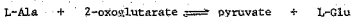
enzyme was stable and the reaction susceptible to kinetic analysis. They found the series of dicarboxylic acids maleate, succinate, glutarate and adipate to have an inhibitory effect which was correlated with a spectral effect on the bound pyridoxal phosphate,⁴ and they calculated dissociation constants for these enzyme inhibitor complexes. They reported that the enzyme was most abundant in mammalian tissue, with the highest concentration in the heart.

A similar study was made in ox heart by Marino et al (1966), who purified the enzyme by heat precipitation, ammonium sulphate fractionation, Alumina C γ gel treatment and chromatography on hydroxysapatite. They then characterized the pure enzyme by zone electrophoresis, ultracentrifugation, light scattering and various other procedures.

Burks et al (1968) have investigated the molecular weight and other properties of the pig heart muscle enzyme. They obtained an enzyme homogenous to ultracentrifugation and to starch gel electrophoresis at pH 9.0. At lower pH's they obtained a number of electrophoretically distinct species. They found the molecular weight to be $78,600 \pm 2,400$. This molecule was found to have two

cofactor binding sites.

Glutamic alanine transaminase catalyses the reaction:



It is called in full: L-Alanine: 2 oxoglutarate amino-transferase, E.C. 2.6.1.2., or, more ~~conv~~ ly, alanine aminotransferase. It is abbreviated as G.P.T. (Glutamic pyruvic transaminase).

The enzyme was crystallized from rat liver by Matsuzawa et al (1968), giving a specific activity of 500-550 μ moles/min./mg. The enzyme contains two pyridoxal phosphate groups and 23-30 titratable sulphhydryl groups per molecule. The amino acid composition was determined and includes 34 half-cystine residues per molecule. Reaction of the first 7-8 SH groups with p-chloromercuribenzoate led to no loss of activity, after which there was loss proportional to the extent of reaction of the next 16 equivalents with the inhibitor. Resolution and partial reconstitution of the apoenzyme was achieved. The spectrum of the pyridoxal form of the enzyme was found to be pH dependent, with an acid peak at 430 $m\mu$ a basic peak at 335 $m\mu$ and an isosbestic point at 380 $m\mu$.

iv. Regulation of Transamination

The roles of G.O.T. and G.P.T. in metabolism are particularly complicated. Together they interconvert α -kg, pyruvate, oxaloacetate, glutamate, alanine and aspartate, functioning at the junction of protein and carbohydrate metabolism. They also supply some of these substances for various synthetic reactions. An oversimplified view is that they act as final common steps in the elimination of nitrogen from the various amino acids, and as a source of the keto acids for the Krebs cycle and for gluconeogenesis. (Knox & Greengard, 1965, P. 269.)

a. Changes with Dietary Intake

High protein diets have been found to raise the hepatic G.O.T. and G.P.T. activity of rats by as much as 650% over that of rats fed on a protein-free diet (Rosen *et al.*, 1959a). It was also shown that the effect was not mediated through stimulated adrenocortical secretion. Elevation of the enzymes was not the result of low carbohydrate content in these diets, as shown by the results of Fitch & Chaikoff (1960). They also showed that the activity of both

enzymes increased only slightly in rats fed on diets containing 60% glucose or fructose.

Starvation for 5 days, which increases nitrogen excretion in the same way as does a high protein diet, caused a several-fold increase in G.P.T. activity and a nearly 100-fold increase in the G.O.T. activity, the effect being more striking in female rats. (Rosen et al., 1959a.)

Awapara (1953) studied the effect of protein depletion on the activity of G.P.T. and G.O.T. in liver, kidney, prostate and seminal vesicles of rat. He found that in the liver G.P.T. activity dropped by 50% in 4 days, with no further drop up to 20 days; there was no effect on G.O.T. There was no effect noticed in the accessory sex organs, whose nitrogen level remained fixed.

The decrease in the levels of the two enzymes with the changes from high protein to protein-free diets, and their elevation with starvation, parallels the well known changes of urea excretion under these conditions, indicating a relationship between transamination and nitrogen excretion which is pertinent to this thesis.

b. Changes due to Hormones

One of the first studies of the effect of glucocorticoids on G.P.T. was that of Tenconi (1960), who showed a significant increase in the activity of G.P.T. in rat livers treated with glucocorticoids, while there was no effect on G.O.T. However, Gavosto et al (1957) found that prolonged treatment with large amounts of cortisone (12mg./100g./day for 3 days) increased the activity of G.P.T. by 59% and that of G.O.T. by 26% in rat liver. The effect of cortisone on hepatic G.P.T. has been confirmed by many workers, whereas that on G.O.T. appears to be variable and often insignificant.

Rosen et al (1959b) studied the response of G.P.T. to cortisone in great detail. They found that the activity increased markedly in thymus, liver and pancreas, and to a lesser extent, in kidney. There was no response in other organs studied. The effects of steroids paralleled their glucocorticoid action and biologically inactive analogs of cortisone were ineffective as inducers. Harding & Rosen (1961)

showed that adrenalectomy lowered liver G.P.T. but did not change the level of G.O.T., while an extension of the work by Rosen (1964) showed that hepatic G.P.T. increased in rats treated with various glucocorticoids, the greatest increase being obtained with cortisol. There was no effect on G.O.T.

Segal et al (1963) showed that the accumulation of G.P.T. after treatment with cortisone was the result of an increased rate of synthesis of the enzyme. However, they postulated that the response of G.P.T. to cortisone is a secondary effect. In other words, cortisone stimulates mobilization of extrahepatic proteins and this causes the increased enzyme level, the changes induced by the hormone in nitrogen metabolism and gluconeogenesis necessitating faster catalysis and thus a rise in the concentration of the enzyme.

Keller et al (1969) found hepatic and pancreatic G.P.T. inducible in rats by corticosteroids, while Dalton & Smart (1970) found that they increased transaminase activity in general in rat liver. Reports by Segal et al (1962) have

shown that G.P.T. induced by cortisone has the same properties as normal G.P.T.

Of the sex hormones, testosterone was shown to have no effect on the G.P.T. activity in rat liver, (Rosen et al, 1959b), nor on the G.P.T. activity of the liver, heart and temporal muscle of castrated guinea pigs. (Kochakian & Endahl, 1956.)

Eckstein & Shain (1963) found that gonadotropin increased the G.O.T. activity of rat ovaries by over 30%, the effect being greatest in one-month old rats but had no effect on the enzymes in the uterus. The effect was not mediated through the pituitary, since hypophysectomized rats showed the same response. Estrogens increased the uterine G.O.T. activity by 25%-30% and did not affect the ovarian enzyme level, although both organs increased in weight. Thus a correlation between organ size and G.O.T. activity was shown with gonadotrophin on ovaries and estrogens on uteri. They also showed that growth hormone increased the size of ovaries,

but that like estrogen, this was not associated with increased G.O.T. levels.

Thyroxine inhibited the response of rat liver G.P.T. to cortisone, although it slightly increased the basal levels. (Rosen et al., 1959b.) Insulin however, had no effect on the basal G.P.T. activity of rat liver, nor on its response to cortisone, while in alloxan diabetic rats the level of G.P.T. was 10 times higher than normal and insulin decreased the enzyme level to the normal range.

Sheid et al (1965) studied the effect of a wide variety of hormones on the levels of rat liver G.O.T. They showed a two-fold increase in activity due to large doses of cortisone (6 daily injections of 12.5 mg.). ACTH gave a smaller increase, while cortisol, thyroxine, growth hormone, oestradiol and testosterone showed no significant change. They also found that injections of L-aspartate gave a slight increase in G.O.T. activity, which was abolished by actinomycin and puromycin, indicating that L-aspartate stimulated overall

protein synthesis. The induction by cortisone was not affected by actinomycin, but was abolished by puromycin, indicating that the effect of cortisone is at the translation level.

A similar survey by Rutman (1968), in various species, investigated the regulatory effect of hydrocortisone and insulin on the synthesis of G.O.T. and G.P.T. in albino rats, pigeons, lizards, frogs (Rana temporaria) and fish. He found that hydrocortisone induced enzyme activity in rat and pigeon but had no effect on the others. Combined injections of insulin and hydrocortisone suppressed induction of enzyme synthesis in these two species.

c. Metabolic Roles of G.O.T. and G.P.T.

Lardy et al (1965) have shown how two isoenzymes of G.O.T. can regulate metabolic processes. They proposed the following roles for the mitochondrial and supernatant isoenzymes of G.O.T.: During gluconeogenesis, pyruvate is carboxylated in liver mitochondria to oxaloacetate.

This does not diffuse out of the mitochondria but can be transaminated by the mitochondrial G.O.T. to aspartate or reduced to malate. Aspartate, α -kg and malate can then diffuse out of the mitochondria. Production of oxaloacetate by the oxidation of malate, or by transamination of aspartate by the supernatant G.O.T. can then occur. Oxaloacetate is then converted to phosphoenol pyruvate and thence to glucose by various glycolytic enzymes also present in the supernatant fraction of the cells.

The conditions under which liver G.F.T. shows greatest and most widely attested increases include treatment with cortisone or ACTH, high protein diet, starvation and alloxan diabetes. The common physiological characteristics of these conditions are enhanced gluconeogenesis, increased protein catabolism, and a rise in the amino acid pool in the liver. The rise in G.P.T. has been seen as both the consequence and the cause of these phenomena by different workers. Knox & Greengard (1965) consider it to be an appropriate assumption that the change in enzyme level is both a response to some primary changes in a physiological state of the tissues, and the

cause of some of the secondary changes. They consider two of the secondary changes resulting from increased levels of these liver transaminases, namely gluconeogenesis and nitrogen excretion, to be the physiological roles of the enzymes.

Weber et al (1963) showed that actinomycin and puromycin inhibit the cortisone-induced elevation of enzymes involved in glycogen metabolism, while Cavosto et al (1957) and Rosen et al (1958) have suggested that the increase in transaminase levels is responsible for increased gluconeogenesis. It is feasible that the increase in transaminase activities is one of the important factors directly responsible for stimulating gluconeogenesis in the liver. The transaminase reactions feed the products of amino acids into the carbon pool, as α -kg, pyruvate and oxaloacetate, from which glucose is made. (Krebs, 1963.) This is accompanied by a loss of nitrogen, linking transamination with protein catabolism, as measured by nitrogen excretion. Elevations of both transaminases and nitrogen excretion occur as a result of high protein diet, cortisone treatment, starvation and alloxan

diabetes, and also as a result of indirect modifications of protein metabolism. Wherever comparisons have been made, the two transaminases change qualitatively in the same way as liver arginase. (Knox & Greengard, 1965, P. 275.)

Since the tissue distributions of G.O.T. and G.P.T. are more extensive than the occurrence of gluconeogenesis or urea formation, they must have other physiological roles, one of which seems to be in connection with organ growth. Some sex hormones which cause organ growth also cause increased transaminase activities. However, during liver regeneration and normal development, the increase in transaminase activities is not striking.

2. Aspartate and Alanine Aminotransferase in Amphibia

1. The Role of Transamination in Urea Formation

A comparative survey of both enzymes in the brain of Rana nigromaculata, mice, catfish and the common turtle was carried out by Imai (1959). He found the levels to be

lower in frog brain than the other species, but did not relate this to the mode of excretion.

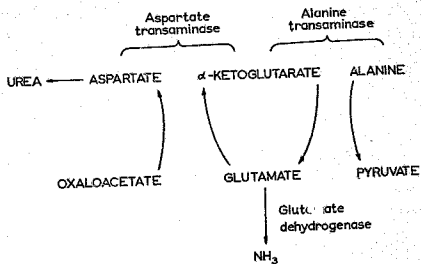
Fiedler & Reichel (1969) investigated both enzymes, among others, in the blood and lymph of Rana esculenta, Rana temporaria and Bufo viridis, and found their activity to be higher than in mammals.

The relationship between the excretory product of vertebrates and transamination was first studied by Hui-Li Chen & Liang-Li (1963) and then by Janssens (1964).

The former workers compared the transamination of twenty-two amino acids in the livers of five vertebrates, viz: rat, pigeon, toad, tortoise and fish. They found the role of transamination to be more important in the protein metabolism of ureotelic and uricotelic animals than in ammoniotelic animals.

As alanine is quantitatively the most important free amino acid in the tissues of Amphibia, (Belinsky, 1970, P. 560), the activities of G.O.T. and G.P.T. assume a special significance in this process, as illustrated in Figure 1b (P. 42). In urea, one nitrogen atom comes from ammonia, while the other is derived from aspartic acid via argininosuccinate. As the diagram shows, if

Fig. 1b.



G.P.T. is active, glutamate (and pyruvate) is produced by transamination of alanine with α -kg; this glutamate is then deaminated by glutamate dehydrogenase and ammonia is excreted. If, however, the glutamate is continuously removed by transamination with oxaloacetate to form aspartate (and α -kg), then urea is the excreted product.

The ratio of the activities of the two enzymes, G.O.T./G.P.T., is therefore important in determining whether alanine is channelled into ammonia or urea, a low ratio indicating that ammonia formation is favoured, while a high ratio indicates the preference for urea excretion. Thus, as one would expect, the ratio of G.O.T./G.P.T. is lower in Xenopus than in ureotelic Amphibia.

Thus a survey of the effect of various experimental and environmental conditions on the ratio of the two enzymes has formed the basis of the present study.

ii. The Effect of Environment on Transamination.

As discussed on P. 4, conditions of water restriction and dehydration cause Xenopus to synthesise urea and store it in the tissues. The African lungfish, Protopterus aethiopicus, which is fully aquatic and ammoniotelic, responds in a similar manner to Xenopus,

surviving long periods of drought by channelling nitrogen into urea and storing it in the tissues until the return of favourable conditions, (Smith, 1930). Janssens (1964) showed that G.P.T. activity decreased ten times from the control level, while G.O.T. activity showed no change. His studies in Xenopus showed similar, although less spectacular results. He measured changes in urea concentration, activities of G.O.T. and G.P.T. in the liver and muscle and the amounts of urea and ammonia excreted in Xenopus kept in 0.9% saline for periods of up to five weeks. The ratio of G.O.T./G.P.T. rose significantly after two weeks, but returned to control values for the remainder of the experiment. It appeared that the increase in activity of G.O.T. relative to that of G.P.T. is associated with the increase in the rate of urea synthesis. This supported Hui-Li Chen & Liang-Li's view (1963) that there is a definite correlation between transamination and urea excretion.

iii. The Effect of Metamorphosis on Transamination.

The change from ammonotelism to ureotelism which occurs in all Amphibian larvae, except Xenopus, has already been discussed, and following from what has been said about the relationship between transamination and urea production, one would expect the ratio of G.O.T./G.P.T. to

rise during metamorphosis concurrently with the change to ureotelism. Wallace (1961) found that the total activity per animal of both G.O.T. and G.P.T. increased after hatching.

The activities of transaminases during later stages of development of Rana catesbeiana were studied by Chan & Cohen (1964). They found a rise in the ratio of G.O.T./G.P.T. at the onset of both spontaneous and thyroxine-induced metamorphosis, which reached a maximum in the liver of the adult frog. The rise was due to a four-fold increase in the activity of G.O.T. while the activity of G.P.T. dropped to slightly below the level of the larval stages.

The activity of G.O.T. in the tail of metamorphosing Rana isponica does not increase, (Yamamoto 1960), whereas in the tail of Xenopus laevis it was found to decrease, while G.P.T. remained constant (Marty & Weber 1968). This indicates that there is a refinement in the control of the enzyme level which permits regulation to be organ specific. (Balinsky, 1970, P. 608.)

C. THE PRESENT STUDY.

The aim of the present work was to study the regulation of the levels of G.O.T. and G.P.T. and especially the ratio of the two enzymes during development, under different environmental conditions and in response to hormone treatment. An attempt was made to correlate the results with the formation of end products of nitrogen metabolism.

D. ASSAY OF THE TRANSAMINATION REACTION.

Most of the early methods of measuring enzymic transamination were manometric. Cammarata & Cohen (1950) measured CO_2 evolved using glutamic decarboxylase to quantitatively decarboxylate the glutamic acid produced by any transaminase reaction. However, the accuracy of this method was limited and more specific spectrophotometric techniques were developed. Thus the absorption of oxaloacetate at 280 $\text{m}\mu$ and of phenylpyruvate and hydroxy-phenylpyruvate at 300 $\text{m}\mu$ allowed for the study of the enzymes transaminating aspartic acid, phenylalanine and tyrosine respectively.

Another technique which was also specific, but not continuous, was used to measure the transamination of aspartate to oxaloacetate and of alanine to pyruvate. The keto acids were reduced using malate and lactate dehydrogenase respectively, in the presence of reduced NAD^+ , the disappearance of which was measured spectrophotometrically at 340 $\text{m}\mu$.

Keto acids and amino acids may be detected by various chromatographic techniques, but this is a tedious procedure as the substrates and products both contain a keto and an amino acid. Thus for accurate results separation

procedures must be carried out and then the results can only be quantitative relative to a standard. Moreover, keto acids are unstable and long incubation periods may cause spontaneous decomposition to compounds which can cause confusion in the interpretation of the chromatogram. Furthermore, substances like α -kg, pyruvate and oxaloacetate are key intermediates in other pathways, and the presence of endogenous glutamic acid necessitates the use of strict controls.

For the present work a colorimetric assay was used by the method of Humoller, Holthaus and Walsh (1957). This method depends on the production of pyruvic acid by G.P.T. directly, and by G.O.T. indirectly, the oxaloacetate produced by this enzyme being decarboxylated to pyruvate by aniline citrate. The pyruvic acid was then converted to its dinitrophenyl hydrazone and assayed spectrophotometrically.

MATERIALS AND METHODS.

A. EXPERIMENTAL ANIMALS.

1. Rats were obtained from the Zoology Department and used to perfect the techniques employed.
2. Xenopus laevis. Adult Xenopus were obtained from the Department of Inland Fisheries, Jonkershoek, Stellenbosch, and kept in fresh water at room temperature. They were fed on raw liver or mince meat once a week and their water changed weekly. They were not fed during the course of the experiments.

Xenopus tadpoles were collected in the field and kept in water at room temperature. They were fed on fish food but were used as soon after capture as possible.

3. Rana angolensis and Rana fuscigula. Adults and tadpoles were caught in the field and kept at room temperature. The adults were kept in glass cages filled with sand and a little water and fed on mealworms. The tadpoles were kept in fresh water and fed on boiled lettuce.

4. Bufo regularis. Adults were obtained in the field. They were kept in glass cages and fed on mealworms.

5. Rana cancrivora. Adults were obtained from Singapore and kept under the same conditions as the Rana angolensis.

B. MEASUREMENT OF THE ACTIVITIES OF ASPARTATE AMINO-
TRANSFERASE AND ALANINE AMINOTRANSFERASE.

1. Preparation of Reagents:

i. Trichloroacetic acid. 100% W/V. 100 g. of TCA was dissolved in 100 ml. distilled water.

ii. Aniline Citrate. 1 g. citric acid was warmed to dissolve it in 1 ml. of distilled water, then 1 ml. of aniline was added. The reagent was prepared freshly each time, since it is unstable and precipitates on standing.

iii. Dinitrophenyl hydrazine reagent. 0.2 g. of 2:4 DNP was dissolved in 20 ml. of concentrated HCl and made up to 100 ml. with distilled water.

- iv. Toluene. Chromatographically pure toluene was used.
- v. Sodium bicarbonate. A 5% solution of NaHCO_3 in distilled water was used.
- vi. Sodium hydroxide. A 10% solution of NaOH in distilled water was used.
- vii. Reflex. In all experiments 0.1M sodium phosphate buffer, pH 7.4 was used.
- viii. Substrates.
- a) Aspartate- α -ketoglutarate in phosphate buffer.
- This was prepared by dissolving 0.665 g. of aspartate, 0.66 g. α -kg (monopotassium salt) and 0.5 g. NaH_2PO_4 in a small volume of water, adjusting the pH to 7.4 with 10% NaOH and diluting the mixture to 25 ml. It was stored in the deep freeze.
- b) Alanine- α -ketoglutarate in phosphate buffer.
- This was prepared as above using 0.445 g. alanine instead of aspartate.

2. Preparation of Homogenates.

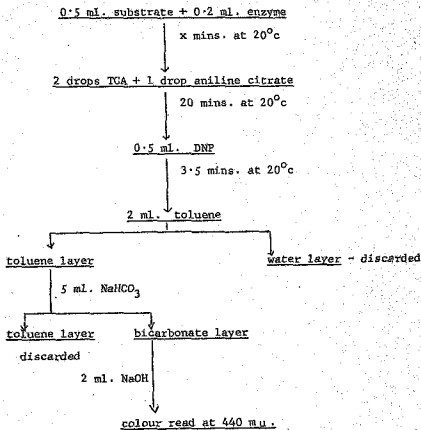
For each experiment a 1/10 W/V homogenate of liver or kidney was prepared in ice cold sodium phosphate buffer, using an Ultra-turrax (18/2) homogenizer. The crude homogenate was centrifuged in polythene tubes in a Servall (SS-1) centrifuge at 10,000 r.p.m. (15,000xg) for 30 mins, at 4°C, (rheostat setting: 170). This sedimented cell debris, mitochondria and nuclei. The supernatant was cleared of fat and diluted with buffer to a final concentration of 1 in 200. Protein concentrations of each homogenate were measured by the Lowry method as described on page 55. Various temperatures and pH's were tested to establish the most suitable conditions for both enzymes. Without exception pH 7.4 and approximately 4°C gave the best activities for both enzymes.

3. Assay Method.

For the assay of both enzymes the method of Humoller, Holthaus & Walsh (1957) was used; (see scheme on page 52). The determination of the transaminase activities depends on the estimation of pyruvic acid, produced directly by alanine aminotransferase and indirectly by aspartate aminotransferase, the oxaloacetate produced in the latter reaction being

SCHEME OF ASSAY METHOD OF HUMOLLER, HOVTHAUS & WALSH.

(described on P. 51)



catalytically converted to pyruvic acid. Pyruvic acid was then converted to its DNP hydrazone, which was estimated colorimetrically:

The incubation mixture contained 143mM amino acid (aspartate or alanine), 9.7mM α -kg, and 91.4mM NaH_2PO_4 in a final volume of 0.7 ml. Thus 0.5 ml. of substrate was used for each experiment, and the reaction was initiated by the addition of 0.2 ml. of the enzyme extract; in the controls 0.5 ml. of buffer was used instead of substrate. The assay mixture was incubated at 20°C for suitable time periods up to 20 mins.

After incubation, 2 drops of 100% Trichloroacetic acid (TCA) were added to stop the reaction, followed by 1 drop of aniline citrate reagent to convert the oxaloacetate to pyruvate. The tubes were well mixed and allowed to stand at 20°C for 20 mins; the time periods, manner and duration of shaking were rigorously controlled to minimise the error due to the numerous extraction procedures involved. Then 0.5 ml. of DNP reagent was added, the tubes mixed and after exactly 3.5 mins. 2 ml. of toluene added to extract the hydrazone of pyruvic acid. The tubes were thoroughly shaken and 1 ml. of each toluene layer transferred to tubes each containing 5 ml. of NaHCO_3 which were again thoroughly shaken. Then 4 ml. of each bicarbonate layer was added

to tubes containing 2 ml. of NaOH and well mixed. A brownish-red colour developed almost immediately and the optical density was read at 440 m μ on a Beckman DB spectrophotometer, using a water blank.

Zero time controls were established by adding the TGA and aniline citrate to the substrate before the addition of the enzyme. All experiments and controls were done in duplicate.

4. Standard Curves.

It was only necessary to construct one standard curve, as in each case the enzyme reaction produced pyruvic acid. A 2.5mM solution of pyruvic acid in 0.1M sodium phosphate buffer, pH 7.4 was prepared. Suitable dilutions were made and colour developed as described under 'Assay Method'.
(Figure 2 P. 73)

5. Reaction Rate Plots.

Assays were carried out at 5 minute intervals between 0 and 30 mins. and the best straight line through the 5 points obtained was drawn to represent the activity of the enzyme.

Activity was expressed in international units, one unit producing one μ mole of keto acid per minute per g. wet weight of homogenate.

Specific activity was defined as activity per mg. of protein, and each figure given represents the mean of at least four experiments (i.e. 4 frogs) done in duplicate; i.e. each point on the graphs represents a minimum of eight optical density readings.

C. ESTIMATION OF PROTEINS.

Proteins were measured by a modification of the method of Lowry, Rosebrough, Farr and Randall (1951), as follows:

Reagent A: 2% W/V Na_2CO_3 in 0.1N NaOH.

Reagent B: 0.5% W/V $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 1% W/V $\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$
(Rochelle's Salt.)

Reagent C: 1 ml. of reagent B in 50 ml. of reagent A.

To 1 ml. of the protein solution was added 5 ml. of reagent C and after 10 mins. 0.5 ml. of IN Folin Ciocalteu reagent, with vigorous shaking. The colour was allowed to develop for 30 mins. and was read at 750 m μ . A standard curve was prepared using bovine serum albumin. (Figure 3, P. 74)

D. HORMONE TREATMENT.

The following hormones were injected into Xenopus laevis adults:

- i. ACTH - 0.1 mg./ml.
- ii. Aldosterone - 0.1 mg./ml.
- iii. Corticosterone - 1.25 mg./ml.
- iv. Insulin - 0.8 units/ml.
- v. Hydrocortisone - 1.25 mg./ml.
- vi. Thyroxine - 1.25 mg./ml.

vii. Triiodothyronine - 1.25 mg./ml.

viii. Vasopressin - 15-20 I.U./ml.

The hormone solutions were injected into the peritoneum every day for four days and the enzyme activities analysed on the fifth day. 2 ml. of the hormone solution was injected per 100 g. frog. Control frogs were injected with water.

The results are shown in Tables I & II, P. 67 and 68.

RESULTS

A. COMPARATIVE SURVEY OF THE EFFECT OF WATER RESTRICTION ON THE LEVELS OF G.O.T. AND G.P.T. IN THE LIVER AND KIDNEY OF XENOPUS LAEVIS.

Xenopus adults were placed on 0.15 M NaCl for 16 days and then returned to fresh water, which was changed weekly over a period of 90 days. The animals were starved for the duration of the experiment, and the activities per unit wet weight of liver of both enzymes were compared with those of control toads, which were kept in fresh water and starved for the same length of time.

If the activities per unit wet weight are considered, liver G.O.T. showed no increase due to water restriction over the control values (Figure 4, P. 7), while G.P.T. in saline-treated animals was somewhat below that of control (Figure 5, P. 70). If specific activity is considered, (Figure 6, P. 77 & 7 P. 70), the levels of both enzymes were shown to increase in the experimental and control animals. This appears to be due to loss of liver protein on starvation (Figure 3B, P. 109), without accompanying loss of activity of the enzymes measured, in other words due to a maintenance of enzyme levels while the level of

Author Goldstein Zoe Rochelle

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